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"Purification process using magnetic particles"
(Puhdistusprosessi magneettipartikkeleita käyttäen)

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PURIFICATION PROCESS USING MAGNETIC PARTICLES

Background of the invention

The present invention relates to the purification of biological substances using magnetic particles which bind the material specifically from a mixture. The invention can be used for instance for purifying nucleic acids DNA or RNA.

Magnetic particles can be coated with a separation reagent which reacts specifically with a desired biological substance. The particles and the bound substance are separated from the mixture and thereafter the substance is released from the particles for further prosecution. Nowadays this is done in practice so that the particles are drawn with a magnet against the wall of the vessel containing the mixture, and the liquid is poured or sucked off the vessel. Thereafter a new liquid can be dispensed into the vessel. Manual or automatic equipments for such separation technology are also commercially available (e.g., Spherotec, Inc., AutoMag Processor (USA), Merck Magnetic Rack (Darmstadt, Germany), PerSeptive Biosystems 96 well plate separator, Multi-6 Separator, Solo-Sep Separator (USA), Dynal Magnetic Particles Concentrators).

The old purification technique for DNA involves ultracentrifugation in a dense cesium chloride gradient. However, also magnetic particle technology described above has been used for purifying nucleic acids.

WO 94/18565 (Labsystems Oy) suggests a method and device for magnetic particle specific binding assay, in which magnetic particles are separated from a mixture by a probe comprising a rod movable in a vertical bore and provided with a magnet at the lower end thereof. The probe is pushed into the mixture with the rod in the lower position, whereby the particles are collected on the probe. Then the probe is transferred to another vessel and the rod is pulled in its upper position, whereby the particles are released. Thus all steps of the assay can be carried out in a separate vessel without having to transfer liquids. In the last vessel, a measurement is carried out.

WO 96/12959 (Labsystems Oy) suggests a magnetic particle transfer tool comprising an elongated body with a concavely tapered tip part. The body further comprises means for providing a longitudinal magnetic field to collect particles to the tip of the body. The magnetic field can be eliminated in order to release the

particles. This tool can be used especially for collecting particles from a large volume and releasing them into a very small volume.

General description of the invention

Now a method according to claim 1 has been invented. Some preferable embodiments of the invention are defined in the other claims.

According to the invention, material to be purified is dispensed in a first medium containing magnetic particles, which have been coated with a binding reagent for the material. The binding reaction takes place, after which the particles are separated and transferred into a second medium, in which a desired further reaction necessary for the purification may take place. The particles can be transferred similarly via further mediums for carrying out further steps of the purification process. All the vessels may contain the necessary reaction medium ready when the particles are released into it.

According to one aspect of the invention, at least one of the mediums contains a surface tension releasing agent. This promotes the complete collection of the particles.

The invention can be used especially for purifying nucleic acids, such as ssDNA, dsDNA, and mRNA.

Brief description of the drawings

The enclosed drawings form a part of the written description.
Figure 1 shows the effect of a detergent in collecting and releasing steps of magnetizable particles.
Figure 2 shows the effect of salt and saccharose in collecting and releasing buffer.
Figure 3 shows the effect of protein in collecting and releasing buffer.
Figure 4 shows the effect of a detergent when magnetic particles of different suppliers were used.

Detailed description of the invention

The invention can be used for instance for the purification of cells, viruses, subcellular organelles, proteins, and especially nucleic acid materials.

The magnetic particles are preferably paramagnetic. The size of the particles is usually less than 50 μm, preferably 0.1 - 10 μm, and most preferably 1 - 5 μm. The

concentration of the particles may be eg. 0.01 - 5 mg/ml, preferably 0.05 - 3 mg/ml, and most preferably 0.2 - 2 mg/ml.

The particles have been coated or treated with a binding reagent, eg. silicon, lectins and/or other reactive functional groups such a oligonucleotides, antibodies, antigens, streptavidin, or biotin.

The particles are preferably transferred from a vessel to another by using a probe comprising a rod movable in a vertical bore and provided with a magnet at the lower end thereof. The probe is pushed into the mixture with the rod in the lower position, whereby the particles are collected on the probe. Then the probe is transferred to another vessel and the rod is pulled in its upper position, whereby the particles are released.

Different kind of surface tension releasing compounds, especially water soluble compounds, can be used in the method. Examples of such are:

A. Tensides, such as
- Soaps
- Detergents; including anionic, kationic, non-ionic and zwitterionic compounds

B. Alcohols, such as
- Polyethylene and polyvinyl alcohols and their protein etc. derivatives

C. Proteins

D. Salts and carbohydrates in high concentrations, such as
- NaCl
- Saccharose

Also mixtures of compounds can be used.

Especially tensides such as detergents are suitable. Preferable detergents are ethoxylated anhydrosorbitol esters. The esters may contain eg. about 4 - 20 ethylene oxide groups.

The concentration of the surface tension releasing compound may be eg. 0.001 - 0.5% (w/v), preferably 0.005 - 0.1% (w/v), and most preferably 0.01 - 0.05% (w/v).

For purification of DNA or mRNA from different sources (for instance, DNA from PCR amplification; DNA from blood, bone marrow or cultured cells; mRNA from eucaryotic total RNA or from crude extracts of animal tissues, cells and plants) the nucleic acids are immobilized by using magnetic particles. The binding can be mediated by the interaction of streptavidin and biotin, whereby particles coated with streptavidin and biotinylated DNA can be used. In addition, DNA can be adsorbed

to the surface of the particles. The binding of mRNA can be mediated by Oligo $(dT)_{25}$ covalently coupled to the surface of the particles.

After the immobilization, the nucleic acids are washed several times to remove all the reaction components resulting from the amplification or other contaminants and, e.g., PCR inhibitors.

The washing can be performed by releasing and collecting complexes in a washing buffer and by transfering the complexes to another well containing fresh washing buffer.

For ssDNA purification the immobilized double-stranded DNA can be converted to a single-stranded by incubation with 0.1 M NaOH and using magnetic separation.

For the isolation of mRNA, it can be eluted from the particles by using a low salt buffer.

The purification process can be performed by a magnetic particle processor, in which all the mediums are ready in separate vessel. A surface tension releasing compound is preferably used in each medium. Suitable disposable plates, such as microtitration plates, comprising the necesary vessels can be used. In one plate, several parallel purifications can be accomplished.

Example of reagents used for a ssDNA purification

1. Particle suspension in eg. phosphate, Tris or Borate buffered saline, pH 7.4, containing 0.1% BSA, 15 mM NaN_3 and eg. 0.02% polyoxyethylene (20) sorbitan monolaurate (Tween 20 ™) as a surface tension releasing agent

2. Binding and Washing buffer (TEN): 10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, eg. 0.02% Tween 20 ™, 15 mM NaN_3, pH 7.5

3. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, eg. 0.02% Tween 20 ™, 15 mM NaN_3, pH 7.5

4. Melting solution: 0.1 M NaOH, eg. 0.02% Tween 20 ™

5. eg. 0.02% Tween 20 ™ in distilled water, 15 mM NaN_3

Example of reagents used for a mRNA direct purification

1. Oligo $(dT)_{25}$ particle suspension in PBS, pH 7.4, containing eg. 0.02% Tween 20 ^{TM} and 15 mM NaN_3

2. Lysis/binding buffer: 100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol (DTT), 15 mM NaN_3, (eg. 0.02% Tween 20 ^{TM})

3. Washing buffer with LiDS (SDS): 10 mM Tris-HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS, 15 mM NaN_3 (eg. 0.02% Tween 20 ^{TM})

4. Washing buffer: 10 mM Tris-HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA, eg. 0.02% Tween 20 ^{TM}, 15 mM NaN_3

5. Elution solution: 2 mM EDTA, pH 8.0 , 15 mM NaN_3, eg. 0.02% Tween 20 ^{TM}

6. Reconditioning solution: 0.1 M NaOH , eg. 0.02% Tween 20 ^{TM}

7. Storage buffer Oligo $(dT)_{25}$: 250 mM Tris-Hcl, pH 8, 20 mM EDTA, 0.1% Tween-20, 15 mM NaN_3

Example of the reagents used for a mRNA purification

1. Binding buffer: 20 mM Tris-HCl, pH 7.5, 1.0 M LiCl, 2 mM EDTA, 15 mM NaN_3 , eg. 0.02% Tween 20 ^{TM}

2. Washing buffer: 10 mM Tris-HCl, pH 8.0, 0.15 mM LiCl, 1 mM EDTA, 15 mM NaN_3, eg. 0.02% Tween 20 ^{TM}

5. Elution solution: 2 mM EDTA, pH 8.0 , 15 mM NaN_3, eg. 0.02% Tween 20 ^{TM}

Example of reagents used for RNA isolation

1. 4 M guanidium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% N-lauryl sarcosine, 0.01 M β-mercaptoethanol

Example of reagents used for a DNA direct purification

1. Particle suspension in Lysis buffer (eg. 50 mM Tris-HCl, pH 7.2, 50 mM EDTA, 3% SDS, 1٧ 2-mercaptoethanol; 50 mM KCl, 10 - 20 mM Tris-HCl, 2.5 mM $MgCl_2$, pH 8.3, 0.5 Tween 20 ^{TM}, 100 μg/ml Proteinase K; 100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 1٧ SDS, 500 μg/ml Proteinase K) containing 15 mM NaN_3

2. Washing buffer containing 15 mM NaN_3 and eg. 0.02% Tween 20 ™

3. Resuspension buffer containing 15 mM NaN_3 and eg. 0.02% Tween 20 ™

Example of the purification process of PCR products by a magnetic particle processor at room temperature

The reagents are dispensed into a subsequent wells of a plate.

Example of a reagent configuration:

Well 1.	Sample (biotinylated DNA, PCR amplicons)
Well 2.	Streptavidin coated magnetic particles in washing buffer
Wells 3 - 5.	Washing buffer
Well 6.	NaOH
Well 7.	TE buffer
Well 8.	Distilled water

Example of processing steps:

Well 2. Mixing, washing and collecting of particles, moving of them into well 3
Well 3. Washing of particles, moving of them into well 4
Well 4. Washing of particles, moving of them into well 1
Well 1. Sample incubation 10', moving of particles into well 4
Well 4. Washing of particles, moving of them into well 5
Well 5. Washing of particles, moving of them into well 6
Well 6. Incubation 5' in melting solution, moving of particles into well 4
Well 4. Washing of particles, moving of them into well 5
Well 5. Washing of particles, moving of them into well 7
Well 7. Rinsing of particles, moving of them into well 8
Well 8. Releasing of particles

The effect of surface tension releasing agent (STRA) in collecting and releasing steps of magnetizable particles

Streptavidin coated magnetic particles (sizes: Scigen streptavidin 3 μm; Scigen; SPHERO™ streptavidin 4 - 4.5μm, Spherotec, Dynabeads M-280 streptavidin 2.8 μm, Dynal) were saturated with biotinylated alkaline phosphatase (Calbiochem, San Diego, CA) for 1 h at +37 °C. Saturated particles were first washed to remove the unbound alkaline phosphatase and were then used to examine the effect of STRA in collecting and releasing steps of a magnetic particle processor. The instrument settings of these exanples were adjusted from 20 μl to 200 μl and the capacity range

of the processor was 1 - 24 samples per run. The processor utilized a rod magnet (cylindrical NdFeB axially magnetized, length 2 mm, width 3 mm) in polypropene tube (outer width 4.5 mm).

Briefly, the particles were processed by releasing and collecting them from well to well so that the whole process comprised of 10 steps. The amount of particles, which remained into the wells after the collection, was estimated with alkaline phosphatase assay. Samples (10 µl) from each well were transferred to an empty microtitration plate (round-bottomed wells, width 6.5 mm).

In this assay alkaline phosphatase saturated particles (0.016 µg - 1 µg particles / 10 µl diluent) were used as standards. Into the wells containing 10 µl samples and standards were added 100 µl pNPP-substrate diluted in diethanolamine (DEA) buffer (Labsystems). The substrate was incubated for 15 minutes at +37 °C with continuous shaking (900 rpm) in Labsystems iEMS Incubator/Shaker. The reaction was stopped by adding 100 µl 1M NaOH into each well and the absorbances at 405 nm were measured by photometer (Labsystems Multiskan).

The amount of remaining particles was determined from a linear standard curve and finally results were expressed as percentage of initial amount of particles (0.2 mg/well).

In Fig 1. is shown the effect of detergent (Tween 20 ™) in different concentrations. The degree of remaining particles (Scigen streptavidin) were over 3% / well, when surface tension releasing agent was not added into the collecting and releasing buffer. When the detergent concentration was ≥ 0.00125%, the particles were collected efficiently.

In Fig 2. is shown the effect of salt and saccharose in collecting and releasing buffer. By adding these components into the buffer, the collection of particles (Scigen streptavidin) was more efficient.

In Fig 3. is shown the effect of a protein (casein) which was improving the collecting steps of particles (SPHERO™ streptavidin) in some degree.

In Fig. 4 is shown the effect of detergent (Tween 20 ™) when the magnetic particles of different suppliers were used.

Claims

1. A process for the purification of a substance, wherein
- material containing the substance, and magnetic particles coated or treated with a reagent which binds the particles to the substance are dispensed in a first medium,
- a binding reaction is let to take place, in which reaction the substance is bound to the particles, and
- the particles and the substance bound to them are separated from the first medium and transferred to a second medium, and if desired, separated from the second medium and transferred to a third medium,

characterized in that
- a surface tension releasing agent is dispensed at least to one of the mediums before the particles are possibly separated from it.

2. A method according to claim 1, wherein the surface releasing compound is a tenside, alcohol, protein, or a salt or carbohydrate.

3. A method according to claim 1 or 2, wherein the concentration of the surface tension releasing compound is 0.001 - 0.5% (w/v), preferably 0.005 - 0.1% (w/v), and most preferably 0.01 - 0.05% (w/v).

4. A method according to any of claims 1-3 for the prurification of cells, viruses, subcellular organelles, proteins, or nucleic acid materials.

5. A method according to any of claims 1-4, wherein the size of the magnetic particles is less than 50 μm, preferably 0.1 - 10 μm, and most preferably 1 - 5 μm.

6. A method according to any of claims 1-5, wherein the concentration of the magnetic particles is 0.01 - 5 mg/ml, preferably 0.05 - 3 mg/ml, and most preferably 0.2 - 2 mg/ml.

7. A method according to any of claims 1-6, wherein the particles are separated from at least one of the mediums by means of a magnetic probe which is pushed into the medium and to which the particles adhere.

8. A method according to claim 7, wherein the surface tension releasing agent is a detergent.

9. A method for separating magnetic particles from a medium, **characterized** in that a surface tension releasing agent is dispensed into the medium before the particles are separated.

10. A medium for use in a purification process of a substance, which medium contains magnetic particles coated with a reagent reacting with the substance, characterized in that the medium contains a surface tension releasing agent.

(57) Abstract

The invention relates to a process for the purification of a substance with magnetic particles treated with a reagent which binds the particles. After a binding reaction i na first medium, the particles and the substance bound to them are separated and transferred to a second medium. According to the invention, a surface tension releasing agent is dispensed at least the mediums before the particles are separated from it. This promotes the complete collection of the particles.

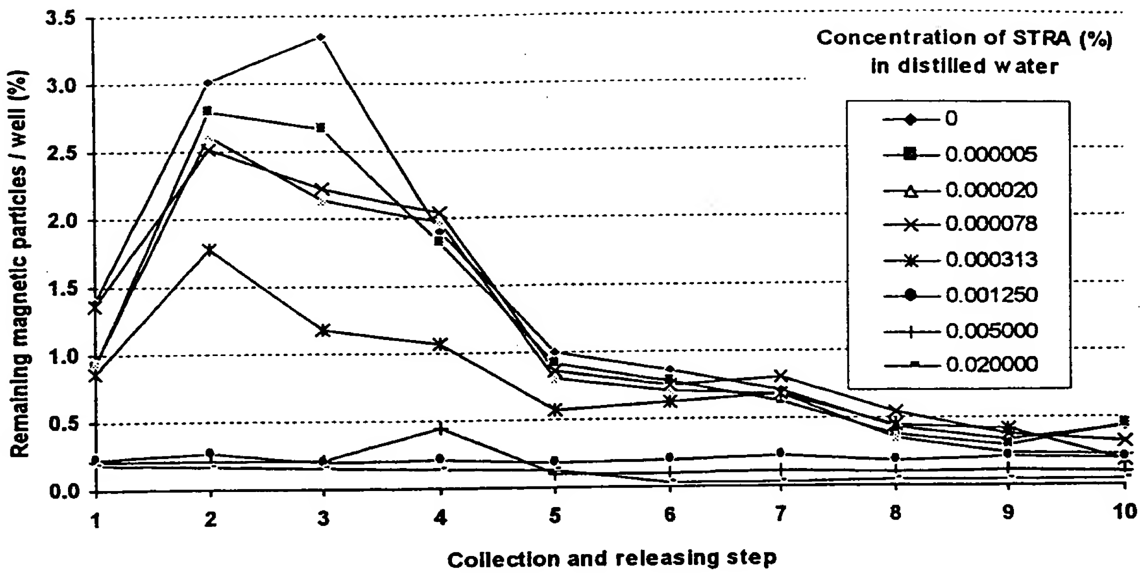

Fig. 1. The effect of surfece tension releasing agent (STRA) in collecting and releasing steps of magnetizable particles

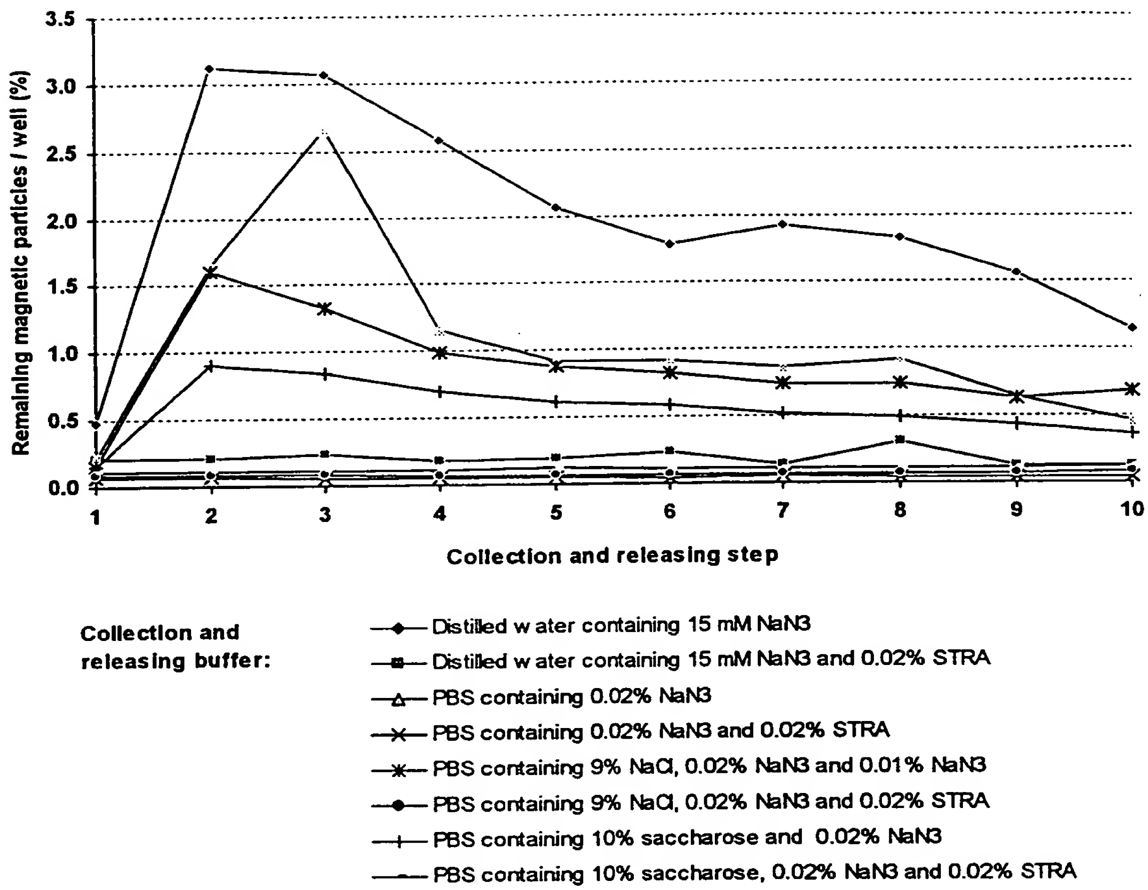

Fig. 2. The effect of salt and saccharose in collecting and releasing buffer.

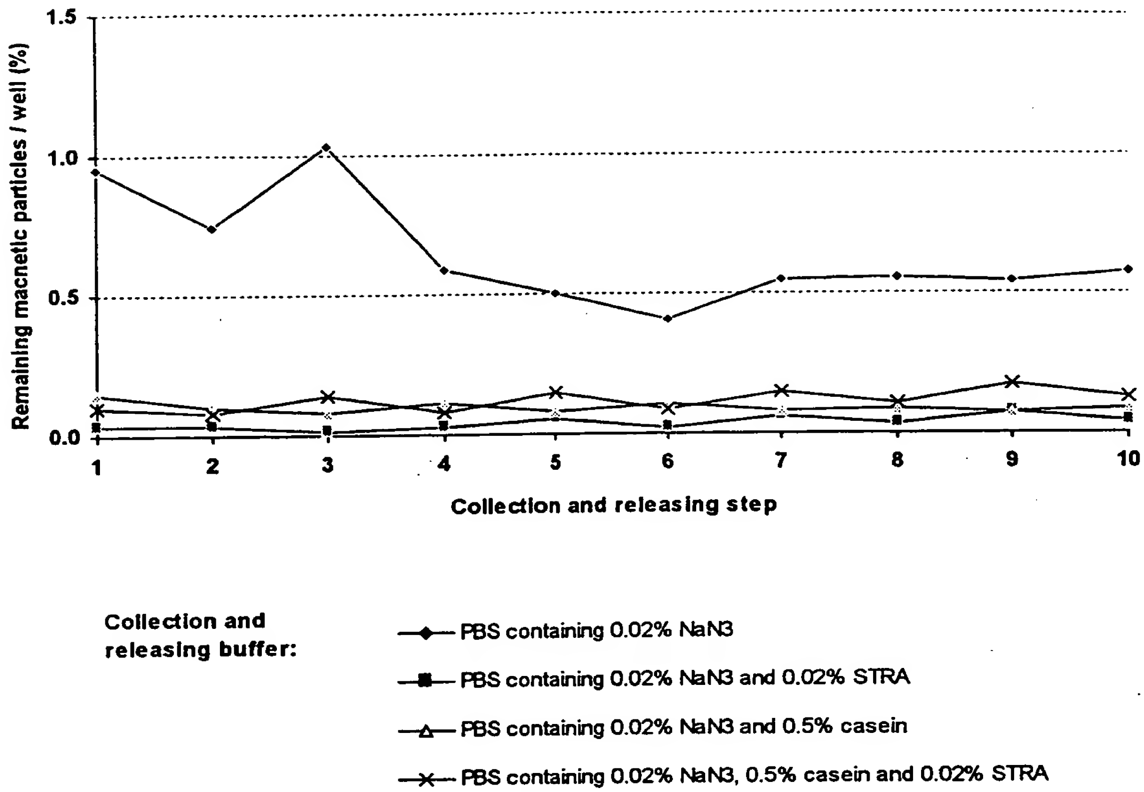

Fig.3. The effect of protein in collecting and releasing buffer.

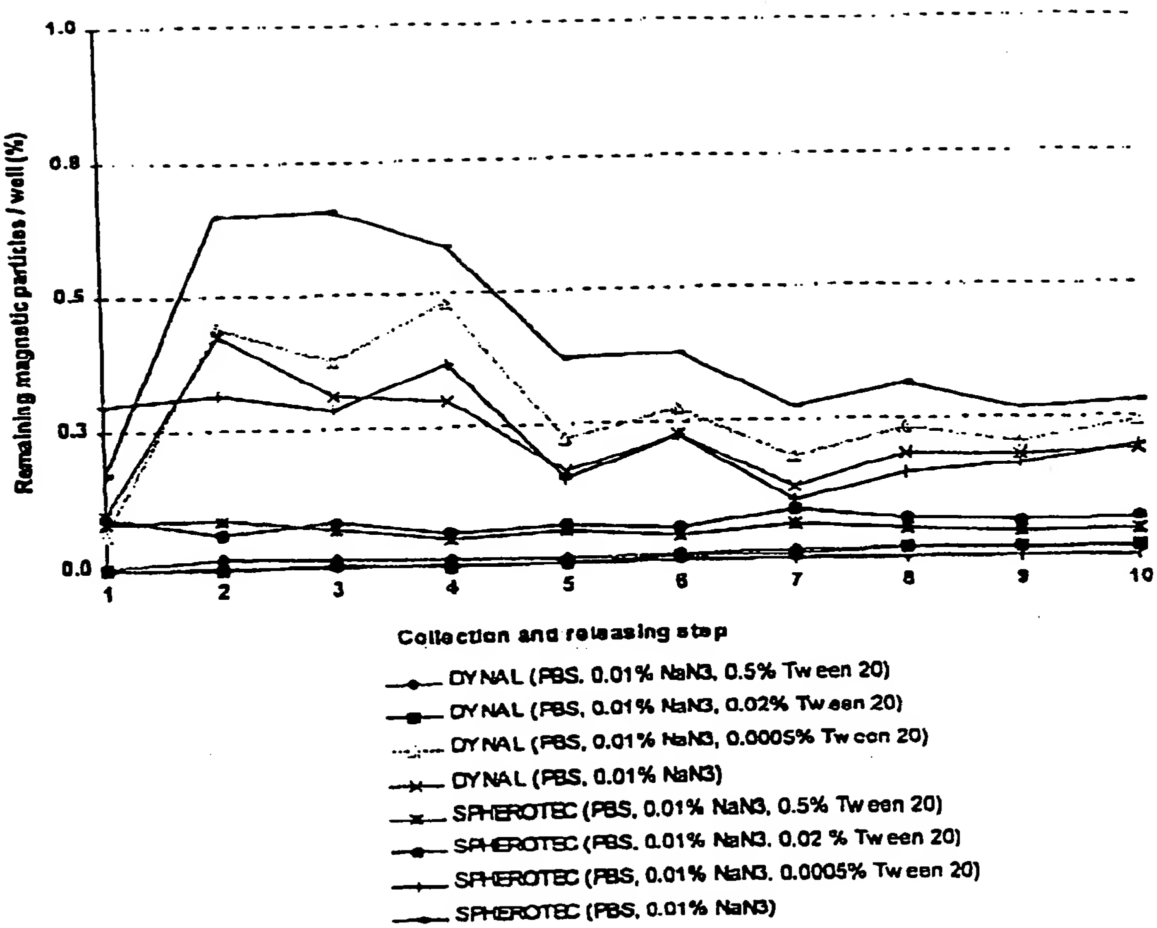

Fig. 4. The effect of detergent (Tween 20 ™) in collecting and releasing steps of magnetizable particles of different suppliers (Dynabeads M-280 Streptavidin, Dynal; SPHERO ™ Streptavidin. Spherotec).

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